MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5618–5626 Vol. 15, No. 10
0270-7306/95/$04.00+0

SCS1, a Multicopy Suppressor of *hsp60-ts* Mutant Alleles, Does Not Encode a Mitochondrially Targeted Protein

YOUMIN SHU AND RICHARD L. HALLBERG*

Department of Biology, Syracuse University, Syracuse, New York 13244

Received 2 May 1995/Returned for modification 27 June 1995/Accepted 17 July 1995

We identified and isolated a *Saccharomyces cerevisiae* gene which, when overexpressed, suppressed the temperature-sensitive phenotype of cells expressing a mutant allele of the gene encoding the mitochondrial chaperonin, Hsp60. This gene, *SCS1* (suppressor of chaperonin sixty-1), encodes a 757-amino-acid protein of as yet unknown function which, nonetheless, has human, rice, and *Caenorhabditis elegans* homologs with high degrees (ca. 60%) of amino acid sequence identity. *SCS1* is not an essential gene, but *SCS1*-null strains do not grow above 37°C and show some growth-related defects at 30°C as well. This gene is expressed at both 30 and 38°C, producing little or no differences in mRNA levels at these two temperatures. Overexpression of *SCS1* could not complement an *HSP60*-null allele, indicating that suppression was not due to the bypassing of Hsp60 activity. Of 10 other *hsp60-ts* alleles tested, five could also be suppressed by *SCS1* overexpression. There were no common mutant phenotypes of the strains expressing these alleles that give any clue as to why they were suppressible while others were not. An epitope (influenza virus hemagglutinin)-tagged form of *SCS1* in single copy complemented an *SCS1*-null allele. The Scs1-hemagglutinin protein was found to be at comparable levels and in similar multiply modified forms in cells growing at both 30 and 38°C. Surprisingly, when localized either by cell fractionation procedures or by immunocytochemistry, these proteins were found not in mitochondria but in the cytosol. The overexpression of *SCS1* had significant effects on the cellular levels of mRNAs encoding the proteins Cpn10 and Mge1, two other mitochondrial protein cochaperones, but not on mRNAs encoding a number of other mitochondrial or cytosolic proteins analyzed. The implications of these findings are discussed.

The *Saccharomyces cerevisiae* chaperonin, Hsp60, is an essential component of the protein refolding machinery located in the mitochondrial matrix (4, 22, 23, 25). While Hsp60, like its *Escherichia coli* counterpart, GroEL (8, 13), appears to play a major role in this refolding process, it does not act alone (16). Genetic (10) as well as biochemical (20) studies indicate that in *E. coli*, there is a protein folding pathway involving the sequential activities and interactions of the proteins DnaK, DnaJ, GrpE, GroEL, and GroES. Homologs of all of these proteins have now been identified in the mitochondria of *S. cerevisiae* (2, 5, 19, 25, 28, 30), and recent evidence (15, 19, 21) suggests that a similar protein folding pathway probably exists within the mitochondrial matrix. It remains, however, to be proven whether this is the case and, more importantly, whether all matrix-targeted proteins follow the same pathway.

One approach that has been used to identify functionally interacting proteins is one in which overproduction of one protein ameliorates, i.e., suppresses, the deleterious effects of a mutant protein with which the overproduced protein is thought to interact. Causing overexpression of a particular gene by introducing extra copies of these genes into conditionally lethal cells (high-copy suppression) has proven successful in identifying and/or confirming the physical interaction of proteins in *E. coli* as well as in *S. cerevisiae.*

As we have now generated an array of 30 different temperature-sensitive (*ts*) alleles of the *S. cerevisiae HSP60* gene (32a), we decided to use high-copy suppression to identify genes whose products functionally interact with Hsp60. Two possible outcomes of this approach were (i) the identification of other, already characterized mitochondrial chaperones that might be allele specific in their suppressing capabilities and (ii) the identification of novel proteins whose functional relationship to Hsp60 has heretofore been unrecognized. This report describes the identification of an example of the latter. We have identified a gene, *SCS1*, encoding a protein of unknown function which, when overproduced, suppresses the *ts* phenotype of some, but not all, of our *hsp60* mutant alleles. However, quite unexpectedly, it apparently does so from an extramitochondrial location. We will present evidence that suggests that Scs1 may be involved in the regulation of expression of genes encoding mitochondrial heat shock proteins and discuss how this might account for its pattern of suppression.

* Corresponding author. Mailing address: Department of Biology, Lyman Hall, Syracuse University, Syracuse, NY 13244. Phone: (315) 443-1104. Fax: (315) 443-2156. Electronic mail address: hallberg@mailbox.syr.edu.

MATERIALS AND METHODS

Yeast strains and growth media. The *S. cerevisiae* strains used in this study are all derived from one of two strains: the diploid **a**/αW303 (*ura3*/*ura3 leu2*/*leu2 his3*/*his3 trp1*/*trp1 ade3*/*ade3*) or αW303. All *hsp60-ts* strains have their chromosomal copy of *HSP60* inactivated by the insertion of a *HIS3* gene (25). The mutant form of *HSP60* in each of these strains (generated either by hydroxylamine treatment [26, 32] or by site-directed mutagenesis [1, 18]) is carried on plasmid pFL39 (a pUC19-derived plasmid containing yeast *CEN4* and *TRP1* sequences; obtained from Patrick Linder).

Cells were grown on YPD (1% yeast extract, 2% dextrose, 2% peptone), YPEG (1% yeast extract, 3% ethanol, 3% glycerol, 2% peptone), or synthetic medium (0.67% yeast nitrogen base, 2% dextrose) supplemented with the appropriate amino acids (0.002%) as required.

Isolation of the multicopy suppressor *SCS1*. The *ts* strain expressing *hsp60-G432D* (12) was transformed with an *S. cerevisiae* genomic library cloned into plasmid YEp351 (*LEU2*) (9). Transformants were allowed to grow on selective plates at 30°C for 3 days and then replicated onto new selective plates and incubated at 38°C until temperature-resistant colonies appeared (5 days). Plasmid DNAs were recovered by standard methods (31) from the four colonies that grew and then restriction enzyme mapped in order to distinguish between YEp351 plasmids and pFL39 plasmids containing the mutant *HSP60* gene. In each case, YEp351 plasmids containing DNA inserts were identified, isolated, and used to transform the original *ts* strain. In only one case did this second transformation result in all transformants becoming non-*ts*. The YEp351 plasmid possessing this suppressing activity contained a 7-kb chromosomal insert. It was designated as pSH1.

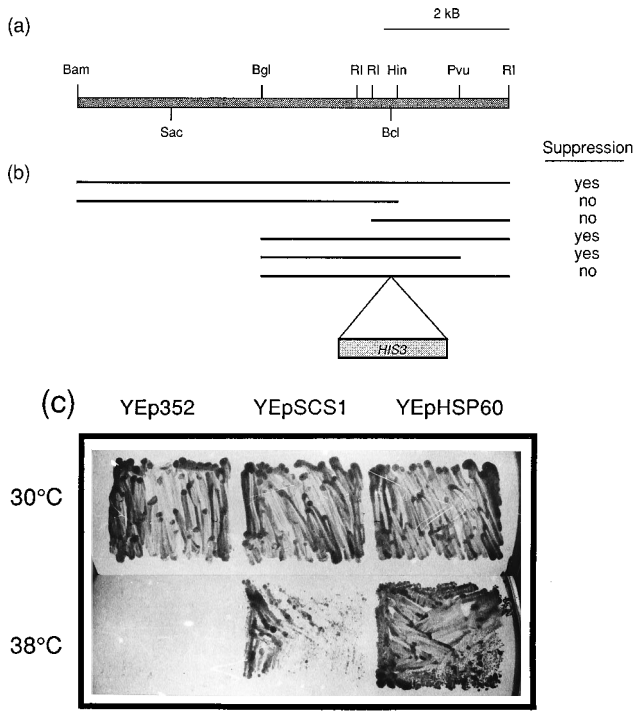

FIG. 1. Identification and mapping of the *SCS1* gene. (a) Restriction map of the 7-kb insert in the YEp351 library that, in high copy number, suppressed the *ts* phenotype of ts3 cells. Bam, *Bam*HI; RI, *Eco*RI; Bgl, *Bgl*II; Pvu, *Pvu*II; Hin, *Hin*dIII; Bcl, *Bcl*I; Sac, *Sac*I. The flanking sites shown are within the plasmid insert itself. There are approximately 100 additional bases of chromosomal DNA between these sites and the multiple cloning site in the plasmid. (b) Determination of partial fragments of the 7-kb insert that possess suppressing activity. The last DNA shown has a 1.7-kb fragment containing the *HIS3* gene inserted at the *Bcl*I site. In each case, the DNA fragment was inserted into YEp352, transformed into the strain expressing *hsp60-G432D*, and tested for its ability to endow cells with the ability to grow at 38°C. (c) Growth at 30 and 38°C of ts3 cells containing different multicopy plasmids: YEp352, YEpSCS1 (YEp352 containing the 3.5-kb *Bgl*II-*Pvu*II fragment shown in panel b), and YEpHSP60 (YEp352 containing *HSP60*) (25).

Plasmid constructions and DNA manipulations. The suppressing gene carried by pSH1 was localized by subcloning restriction fragments of the yeast chromosomal insert (Fig. 1) into the high-copy-number vector YEp352 (*URA3*) (14). These subcloned fragments were introduced into the original strain (ts3) expressing *hsp60-G432D*, and non-*ts* transformants were identified. For sequencing purposes, the 4.0-kb *Bgl*II-*Eco*RI fragment (Fig. 1) was subcloned into the pBS(+) and pBS(−) phagemids. Nested deletions were generated by using exonuclease III digestion. Both strands of the insert were sequenced by standard methods.

Two different protocols were used to create *SCS1*-null strains. Initially for both, a 5.5-kb *Sac*I-*Pst*I (the latter from the multiple cloning site of pSH1) fragment was subcloned into the vector pBS(+) to give plasmid pSH2. Then, in the first case, a 1.7-kb *Bam*HI fragment carrying a *HIS3* gene was cloned into the *Bcl*I site of this new plasmid to create an insertion within the coding region of *SCS1*. In the second case, the same 1.7-kb *HIS3* fragment was cloned into a *Bgl*II-*Bcl*I-cut pSH2 to create an insertion construct in which approximately one-half of the *SCS1* coding region and 610 bases 5′ of the first AUG codon were deleted. Subsequently, *Eco*RI digestion was used to liberate either a linear 3.9-kb or a linear 5.3-kb disrupted *SCS1* gene, each of which was then used in the one-step gene disruption procedure (29) to transform the wild-type **a**/αW303 diploid strain. To obtain haploid *SCS1*-null cells, diploid transformants were induced to sporulate at 25°C, and His^+ colonies were isolated. To confirm that disruption of the *SCS1* gene by each DNA construct had occurred, Southern analyses were carried out on *Eco*RI-digested DNA from all strains, using the 2.2-kb *Eco*RI-*Eco*RI fragment as a probe. In every case, the DNA fragments of the predicted sizes were found for disrupted and nondisrupted forms of *SCS1* (data not shown). The gene containing just the *HIS3* insertion was designated *scs1*::*HIS3(1)*. The gene with the partial deletion plus *HIS3* insertion was designated *scs1*::*HIS3(2)*. Strains expressing the first gene were designated SCS1-null[a]; strains expressing the latter were designated SCS1-null[b].

To generate *SCS1* genes that expressed proteins carrying a foreign epitope, a 112-bp *Bgl*II fragment encoding three repeats of the hemagglutinin (HA) peptide (YPYDVPDYA) was inserted internally or C terminally into the *SCS1* gene. In the first case, the 112-bp fragment was directly cloned into the *Bcl*I site. Such a construct introduced the 27 amino acids in the same reading frame as Scs1. The gene encoding this protein was designated *SCS1*::HA_3*(1)*. For the C-terminal epitope tagging, a *Bgl*II site had to be introduced at the carboxyl terminus of the coding sequence. To generate single-stranded DNA for site-directed mutagenesis, the 3.3-kb *Bgl*II-*Pvu*II fragment containing the entire *SCS1* gene was subcloned into the shuttle vector pRS315 (33), which is also a phagemid. A synthetic oligonucleotide (GACAGCGAGATACAAGATCTATGAAACAGCAATTCAT) was designed such that a *Bgl*II site was created immediately in front of the termination codon. Site-directed mutagenesis was carried out by the method of Kunkel et al. (18), and the correctly mutagenized clones were identified by digesting isolated DNAs with *Bgl*II. A 112-bp *Bgl*II fragment containing the HA epitopes was cloned into the newly generated *Bgl*II site of each of these plasmids. Restriction analysis was used to identify plasmids into which a single insertion occurred in the proper orientation. The gene encoding this terminally tagged protein was designated *SCS1*::HA_3*(2)*.

The fusion gene that expressed an Scs1 with a terminal tail of histidine residues (*SCS1*::his_6) was created by site-directed mutagenesis. A synthetic oligonucleotide (GACAGCGAGATACAGCACCACCACCACCACCACTGAA ACAGGAATTCATTAC) was used to introduce a sequence encoding six histidine residues at the carboxyl terminus of Scs1 by the methods described above. To purify Scs1-His_6, cells expressing such a fusion protein were grown in synthetic medium and harvested in late log phase. The cells were resuspended in lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 Tris-HCl [pH 8.0]) and broken by using glass beads. Scs1-His_6 was purified through an Ni column supplied by Qiagen, using the procedure suggested by the company.

RNA isolation and analysis. Mid-log-phase cells growing in synthetic medium at 30°C were divided into two equal parts. One part was shifted to 38°C, while the other was kept at 30°C. Following a 30-min incubation, cells were harvested and total RNA was isolated by the hot phenol method (17). RNA treated with glyoxal (as described in reference 25) was electrophoretically separated on 1% agarose gels and then transferred to Biotrans membranes. Cloned copies of specific genes, liberated from their respective plasmids by nuclease digestion, were isolated and labeled with [^{32}P]dATP by random priming (1). Northern (RNA) analyses was carried out on the Biotrans membranes with these radioactive probes.

Western blot (immunoblot) analyses and cell fractionation. To identify HA epitope-tagged forms of Scs1 in whole cell extracts, pelleted yeast cells were dissolved in 1.4 M NaOH–5% β-mercaptoethanol, and the solubilized proteins were processed as previously described (12). These proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% gels), transferred to nitrocellulose membranes, and immunodecorated with a monoclonal antibody directed against the HA epitope (12CA5; Boehringer Mannheim) followed by an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibody (Bethesda Research Laboratories).

For cellular fractionation, late-log-phase cells growing in synthetic medium were harvested, spheroplasts were generated, and cell homogenization was carried out as described by Daum et al. (6). Initial homogenates were centrifuged at 3,500 × *g* for 5 min to remove partially and unbroken cells. The resulting supernatant was considered to be essentially an unfractionated whole cell homogenate. For fractionation purposes, this whole cell homogenate was spun at 20,000 × *g* for 15 min to pellet nuclei and mitochondria. The supernatant generated was designated the postmitochondrial fraction. After the nuclear/mitochondrial pellet was redissolved in initial homogenization buffer, aliquots of the three solutions were added to a 0.5 volume of 3× SDS-gel sample buffer and subjected to SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes and immunodecorated as described above.

Indirect immunofluorescence. To visualize cells immunocytochemically, the procedure described by Davis and Fink (7) was followed except that zymolyase was used to generate spheroplasts and protease inhibitors were omitted. The primary antibody used was the monoclonal antibody described above directed against the HA epitope (final concentration, 1 μg/ml), and the secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (final concentration, 5 μg/ml; Bethesda Research Laboratories).

RESULTS

Identification of a high-copy-number suppressor of a conditional *hsp60* mutant. To identify other genes which, when overexpressed, suppress the *ts* phenotype of *hsp60* mutants, we transformed strain ts3, expressing *hsp60-G432D*, a strong *ts* allele (12), with a high-copy-number plasmid (YEp351) yeast genomic DNA library. The resulting transformants, initially selected at 30°C, were then screened at 38°C. Among approximately 30,000 transformants, four colonies grew at 38°C after 4 days of incubation. To determine if suppression of the *ts* phenotype of ts3 cells was due to a plasmid-borne gene, plas-

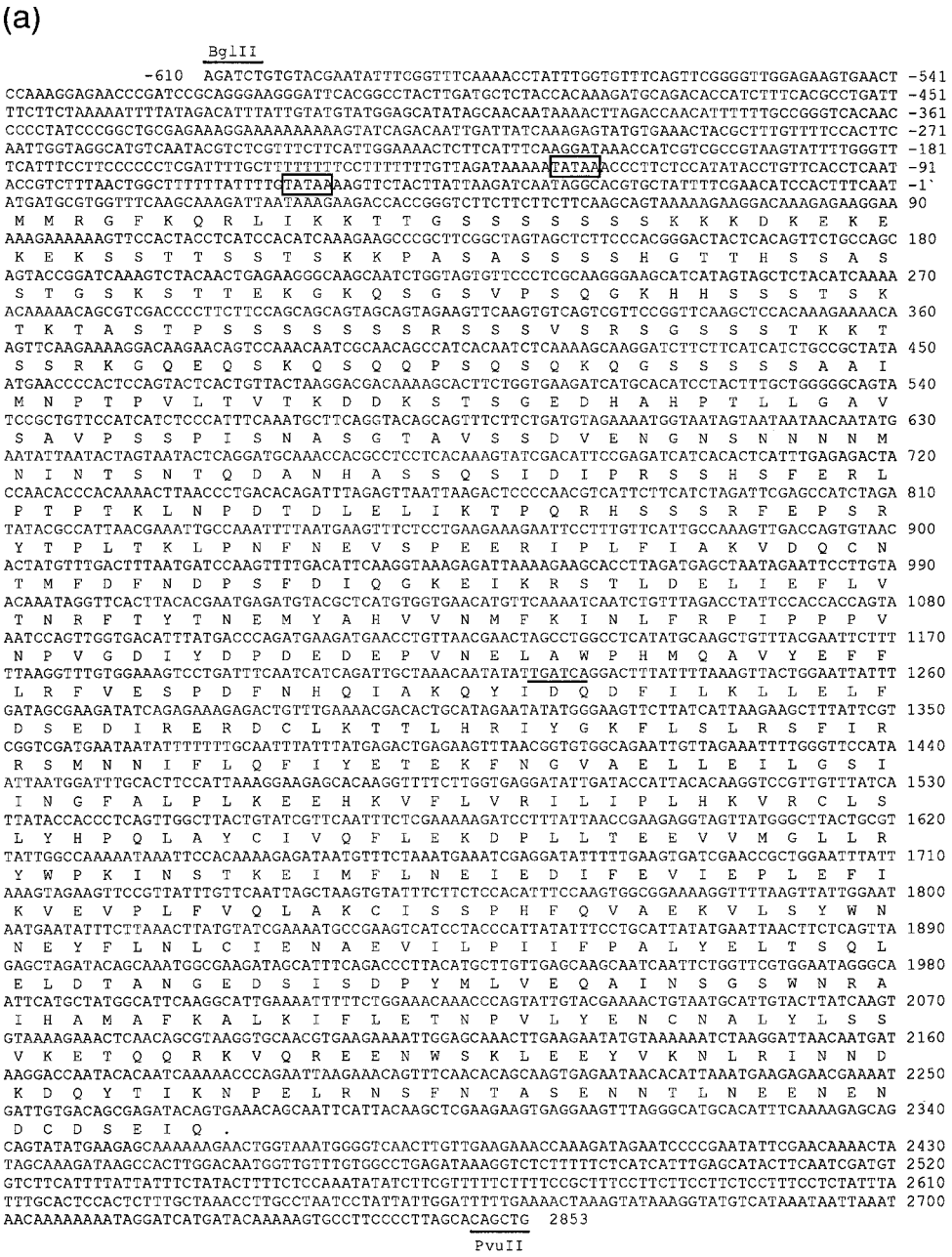

FIG. 2. Sequence analysis of *SCS1*. (a) Sequence of the suppressing *Bgl*II-*Pvu*II DNA fragment. The single long open reading frame is shown. Putative TATA boxes at about −120 and −70 bases 5′ of the AUG start codon are indicated. The underlined *Bcl*I sequence is the site at which the *SCS1* coding sequence was interrupted by insertion of a *HIS3* gene (see Fig. 1). (b) Sequence comparisons between Scs1 and a protein encoded by a partial cDNA isolated from human myeloblasts (GenBank entry D26445). The upper sequence is that of the yeast protein (S.c.) from amino acid residues 276 to 712 (from a total of 757); the lower is the human protein (H.s.) from amino acid residues 26 to 445 (from a total of 484). The middle sequence indicates identities between the two with letters and conservative changes with plus signs. Dashes indicate gaps. Not considering the sequence in the yeast protein with no counterpart in the human protein, the sequences of these two protein fragments exhibit 60% identity and 76% similarity when conservative changes are counted.

mid DNAs were isolated from each of the non-*ts* cell lines and retransformed into the original host. Of the four different plasmids, only one conferred all new ts3 transformants with the ability to grow at 38°C, indicating that this suppression was the result of a plasmid-borne gene. This suppressing plasmid, containing a 7-kb chromosomal insert (Fig. 1a), did not contain the *HSP60* gene (which would have suppressed by complementation), since the restriction map of the plasmid insert differed from that of the bona fide *HSP60* gene (25). From this finding, we concluded that an extragenic suppressor had been isolated, and we named it *SCS1* (suppressor of chaperonin sixty-1).

Different-size fragments of the 7-kb chromosomal insert in YEp351 were subcloned into another high-copy-number plasmid (YEp352), and their abilities to suppress were determined (Fig. 1b). The suppressing activity was localized to a 3.5-kb fragment. An insertion of a 1.7-kb *HIS3* gene into a *Bcl*I site within this 3.5-kb segment also abolished the suppressing activity, indicating that the *Bcl*I site is in the open reading frame

(b)

```
S.c. LPNFNEVSPEERIPLFIAKVDQCNTMFDFNDPSFDIQGKEIKRSTLDELIEFLVTNRFTYTNEMYAHVVN
     L +  +V P ++  LFI K+ QC  +FDF+DP  D++ KE+KR+ L E++E++  NR   T  +Y  VV+
H.s. LLHIRDVPPADQEKLFIQKLRQCCVLFDFSDPLSDLKWKEVKRAALSEMVEYITHNRNVITEPIYPEVVH

S.c. MFKINLFRPIPPPVNPVGDIYDPDEDEPVNELAWPHMQAVYEFFLRFVESPDFNHQIAKQYIDQDFILKL
     MF +N+FR +PP  NP G  +DP+EDEP  E AWPH+Q VYEFFLRF+ESPDF   IAK+YIDQ F+L+L
H.s. MFAVNMFRTLPPSSNPTGAEFDPEEDEPTLEAAWPHLQLVYEFFLRFLESPDFQPNIAKKYIDQKFVLQL

S.c. LELFDSEDIRERDCLKTTLHRIYGKFLSLRSFIRRSMNNIFLQFIYETEKFNGVAELLEILGSIINGFAL
     LELFDSED RERD LKTTLHRIYGKFL LR++IR+ +NN IF +FIYETE NG+AELLEILGSIINGFAL
H.s. LELFDSEDPRERDFLKTTLHRIYGKFLGLRAYIRKQINNIFYRFIYETEHHNGIAELLEILGSIINGFAL

S.c. PLKEEHKVFLVRILIPLHKVRCLSLYHPQLAYCIVQFLEKDPLLTEEVVMGLLRYWPKINSTKEIMFLNE
     PLKEEHK+FL+++L+PLHKV+ LS+YHPQLAYC+VQFLEKD  LTE VVM LL+YWPK +S KE+MFLNE
H.s. PLKEEHKIFLLKVLLPLHKVKSLSVYHPQLAYCVVQFLEKDSTLTEPVVMALLKYWPKTHSPKEVMFLNE

S.c. IEDIFEVIEPLEFIKVEVPLFVQLAKCISSPHFQVAEKVLSYWNNEYFLNLCIENAEVILPIIFPALYEL
     +E+I +VIEP EF+K+  PLF QLAKC+SSPHFQVAE+ L YWNNEY ++L  +NA  ILPI+FP+LY
H.s. LEEILDVIEPSEFVKIMEPLFRQLAKCVSSPHFQVAERALYYWNNEYIMSLISDNAAKILPIMFPSLY--

S.c. TSQELDTANGEDSISDPYMLVEQAINSGSWNRAIHAMAFKALKIFLETNPVLYENCNALYLSSVKETQQR
                                      WN+ IH + + ALK+F+E N  L+++C   + +   + + +
H.s. ----------------------RNSKTHWNKTIHGLIYNALKLFMEMNQKLFDDCTQQFKAEKLKEKLK

S.c. KVQREENWSKLEEYVK
       +REE W K+E   K
H.s. MKEREEAWVKIENLAK
```

FIG. 2—*Continued.*

of *SCS1* or is in its control region. While overexpression of *SCS1* allowed ts3 cells to grow at 38°C, it was not as effective as *HSP60* itself in ameliorating this temperature sensitivity (Fig. 1c).

***SCS1* encodes an evolutionarily conserved protein with an as yet unknown function.** The entire sequence of the 3.5-kb *Bgl*II-*Pvu*II DNA fragment was determined (Fig. 2a). It contained a single long open reading frame of 2,271 bases potentially encoding an 86-kDa protein of 757 amino acid residues. The position of this reading frame as encoding *SCS1* was consistent with the DNA fragments that did or did not suppress (Fig. 1b). Two putative TATA boxes were found 120 and 70 bases upstream from the first ATG codon. As determined by a search of the Prosite database, the translated protein sequence contained no obvious functional motifs. One notable feature of the protein sequence was the abundance of serines and threonines in the first 200 amino acids, where they account for about 20% of the total amino acid residues.

Using both a BLASTP and a TBLASTN search of the National Center for Biotechnology Information database, we identified several homologous protein sequences. The first was an *S. cerevisiae* sequence essentially identical (two amino acid differences) to the sequence of Scs1. The information accompanying this sequence (not published) in the database (GenBank entry U06330) indicated that it encoded a multicopy suppressor of a mutant allele of *ROX3* (27), a gene encoding a predominantly nuclear protein that regulates the expression of the iso-2-cytochrome *c* gene, *CYC7*. Another database sequence to which *SCS1* was related was that of a partial cDNA created from human myeloblast mRNA (GenBank entry D26445). In this case, the 484-amino-acid human sequence encoded by this partial cDNA was homologous throughout almost its entire length to the C-terminal two-thirds of the Scs1 sequence (Fig. 2b). The overall identity was 55%, with a 78% similarity when conservative substitutions were considered. In addition, amino acid sequences of from 26 to 200 residues from short partial cDNA sequences from mice, rice, and *C. elegans* genes and a second human gene gave identities to Scs1 ranging from 63 to 69% (data not shown). No function was ascribed to any of these homologs.

***SCS1* can suppress other *hsp60-ts* alleles but is not a bypass suppressor.** An important question was whether *SCS1* suppressed in an allele-specific manner. If *SCS1* is not allele specific in its suppression, and if it suppresses a group of *hsp60-ts* alleles that elicit similar mutant phenotypes, we might be able to predict what sort of functional defect overproduction of Scs1 corrects. Therefore, 10 *hsp60-ts* mutant strains were transformed with YEpSCS1, and the transformants were tested for growth at 38°C (Fig. 3). Among the 10 strains transformed, 5 had their *ts* phenotypes suppressed. Thus, *SCS1* is not allele specific. Comparing the *ts* phenotypes of these strains, such as the induction of solubility of Hsp60 (11, 12) at a high temperature, the reversibility of high-temperature treatment on viability, or the buildup of precursor forms of mitochondrial proteins at the nonpermissive temperature, we found only two strains, those expressing *hsp60-Y510D* and *hsp60-ts8*, to have phenotypes similar to that of ts3 cells. Thus, high-copy suppression of all of these strains did not seem to be occurring by the alleviation of one particular Hsp60 misfunction. A possible explanation for this finding will be discussed below.

As not all mutant strains are suppressed by the overproduction of Scs1, it is unlikely that Scs1 replaces the function of heat-inactivated Hsp60; that is, it does not act as a bypass suppressor. However, to test that possibility directly, we introduced the *SCS1* gene on YEp351 into a strain whose chromosomal copy of *HSP60* was disrupted but which was viable because it carried a copy of *HSP60* on YEp352, a noncentromeric, *URA3*-containing plasmid. Such double transformants were grown in media that selected for retention of YEp351 (and *SCS1*) but applied no selection for YEp352 (and *HSP60*). After several days of growth, cells were plated on YPD agar, and after colonies had formed, they were replicated onto 5-fluoro-orotic acid-containing YPD plates to see if any cells had lost the YEp352 plasmid and could therefore survive without a copy of a functional *HSP60* gene. No cells grew on 5-fluoro-orotic acid at either 25 or 37°C (data not shown), indicating that overexpression of *SCS1* does not allow cells to dispense with Hsp60 and showing that suppression must take place by ameliorating the defect in Hsp60 function at high temperatures rather than by bypassing this defect.

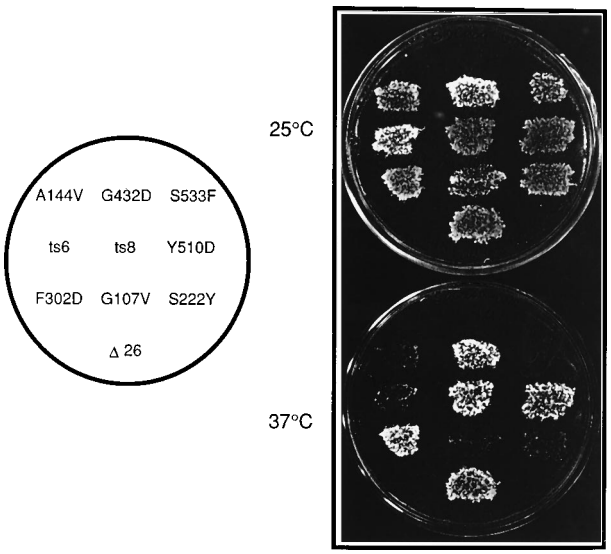

FIG. 3. *SCS1* suppression of strains expressing other *hsp60-ts* alleles. Cells expressing the alleles indicated in the diagram on the left were transformed with plasmid YEp351 containing the 7-kb chromosomal insert. (Mutant strains are designated by [i] the altered amino acid in *HSP60* [e.g., Y510D], [ii] the absence of a designated number of amino acids from the carboxyl terminus of Hsp60 [Δ26], and [iii] *hsp60-ts* alleles whose alterations have not yet been determined [ts8 and ts6]. In all strains tested, the chromosomal copy of *HSP60* is *hsp60*::*HIS3* and the mutant *HSP60* allele is carried on pFL39 [12].) Such transformants were grown at 25°C on selective plates in order to maintain plasmid YEp351, and then they were replicated to two new selective plates. One of these plates was maintained at 25°C the other was incubated at 37°C.

If some mutant Hsp60s were not totally inactivated at 38°C and consequently had insufficient activity to maintain normal mitochondrial biogenesis, this deficiency could conceivably be overcome by increasing the levels of Hsp60 within mitochondria. Western analyses of both suppressed and nonsuppressed mutant strains, as well as wild-type controls, at both 30 and 38°C revealed (data not shown) no obvious differences in Hsp60 amounts other than the normal increases in Hsp60 that occur at higher temperatures (22, 25). This lack of effect of *SCS1* overexpression on the expression of *HSP60* was independently confirmed by Northern analyses (see below).

***SCS1* is not essential at 30°C but is required for cell survival at elevated temperatures.** The chromosomal copy of *SCS1* was inactivated in both haploid and diploid cells by one-step gene disruption (29) using a linear copy of *SCS1* into which a *HIS3* gene was inserted [*scs1*::*HIS3(1)*; see Materials and Methods]. In both cases, viable His^+ cells that grew at 30°C on glucose-containing medium were obtained. DNA from both the haploid and diploid transformants was analyzed, and this analysis confirmed that a copy of the *SCS1* gene had been disrupted with the *HIS3* gene (data not shown). Thus, it appeared that *SCS1* was not essential for growth at 30°C. Haploid cells expressing only *scs1*::*HIS3(1)* (SCS1-null[a] cells) grew on nonfermentable carbon sources, but regardless of the growth medium, SCS1-null[a] cells grew more slowly than wild-type cells at 30°C (Table 1). SCS1-null[a] cells failed to grow at 38°C irrespective of the growth medium (Fig. 4). In examining a number of other growth-related properties of SCS1-null[a] cells, we found that in addition to being *ts*, they were also hypersensitive to ethanol (Table 1). Diploid cells [*SCS1*/*scs1*::*HIS3(1)*] were indistinguishable from wild-type cells with regard to their growth properties (data not shown).

To confirm that the *ts* phenotype was the result of the disrupted *SCS1* gene, a wild-type *SCS1* gene on the centromere-containing plasmid pRS315 (33) was reintroduced into SCS1-null[a] cells. Such transformants were no longer *ts* (Fig. 4).

TABLE 1. Growth characteristics of cells expressing a nonfunctional *SCS1* gene

Cell type[a]	Doubling time[b] (h)			Relative growth on ethanol[c]			
	YPD	YPGal	YPEG	0%	3%	6%	9%
W303	2.0	2.8	2.5	1 (13.6)	0.71	0.11	0.02
SCS1-null[a]	3.1	2.9	4.9	1 (9.7)	0.07	0.04	0.02

[a] W303 cells (see Materials and Methods) are the parent strain of those in which *SCS1* has been disrupted by insertion of a functional *HIS3* gene. These latter cells contain the gene *scs1*::*HIS3(1)* and are designated SCS1-null[a].

[b] The compositions of the three media in which cells were grown are given in Materials and Methods. All growth measurements were made at 30°C.

[c] Cells growing in YPD at 30°C were diluted to an optical density at 600 nm (OD_{600}) of 0.1 into fresh YPD medium containing ethanol at various concentrations. The cultures were incubated at 30°C, and the OD_{600} of each culture was measured 16 h later. The relative amount of growth in a particular concentration of ethanol was determined by dividing the OD_{600} of that culture by the OD_{600} of the comparable culture containing no ethanol. Each number in parentheses is the OD_{600} of the control culture at 16 h.

As the initial disruption of *SCS1* was generated by simply inserting a *HIS3* gene into it, it was conceivable that the *ts* phenotype of SCS1-null[a] cells was due to the expression of a truncated form of Scs1 that simply could not function at elevated temperatures. To rule out this possibility, we generated a second *SCS1* disruption strain in which 610 bases 5′ of the first AUG codon and two-thirds of the *SCS1* open reading frame were replaced with a *HIS3* gene [*scs1*::*HIS3(2)*; see Materials and Methods]. These SCS1-null[b] cells exhibited the same phenotype as the SCS1-null[a] cells, indicating that the *ts* phenotype was due to the absence of Scs1, not the presence of a *ts* form.

One possible reason that *SCS1*-null cells were *ts* (or ethanol sensitive) was that Scs1 is a stress-inducible protein whose function is primarily or solely required at high temperatures or under other stressful conditions and is expressed only then. The experiments described below show that expression of the *SCS1* gene is not markedly affected by heat stress and that the amount and form of Scs1 is similarly unaffected.

Determining the metabolism and cellular location of Scs1. To study the metabolism of Scs1 and ascertain its cellular location, we created a gene encoding an epitope-tagged form of Scs1 (see Materials and Methods for details). This gene, *SCS1*::HA_3*(2)*, was introduced on the single-copy plasmid pRS315 into SCS1-null[a] cells. In both cases, transformants were no longer *ts* (Fig. 5a), indicating that the gene encoding

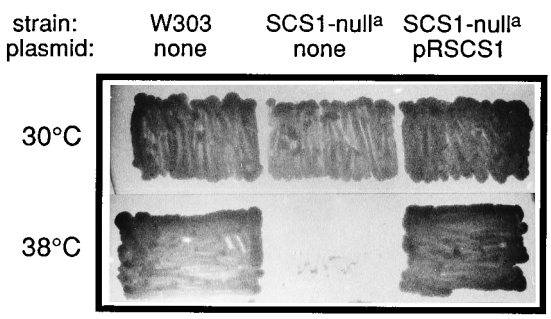

FIG. 4. Growth properties of SCS1-null[a] cells. Wild-type cells, SCS1-null[a] cells containing no plasmid, or SCS1-null[a] cells transformed with pRSCS1 (pRS315 containing *SCS1*) were grown on YPD at 30 or 38°C.

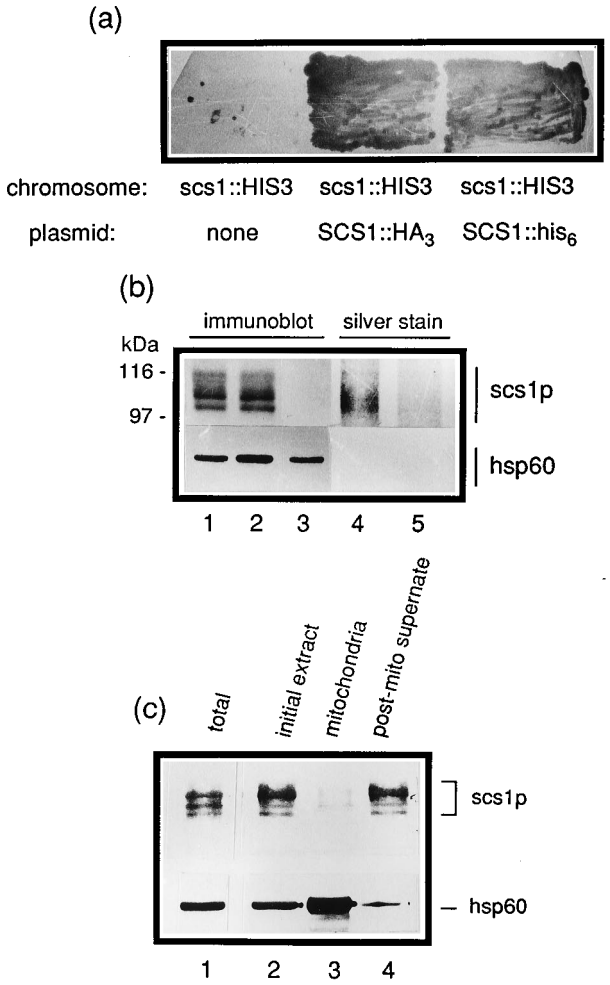

FIG. 5. Analysis of Scs1 fusion proteins expressed in SCS1-null[a] cells. *SCS1* genes containing a C-terminal HA_3 epitope [*SCS1*::*HA*$_3$*(2)*] or a C-terminal hexahistidine sequence (*SCS1*::*his*$_6$) (see Materials and Methods) were introduced into SCS1-null[a] cells on the *CEN* plasmid pRS315. (a) Transformed and nontransformed SCS1-null[a] cells grown at 38°C. (b) Electrophoretic analysis of the fusion proteins. SCS1-null[a] cells expressing *SCS1*::*HA*$_3$*(2)* were grown at 30°C and harvested (lane 1) or transferred to 38°C for 90 min and then collected (lane 2). Total proteins were directly extracted from unbroken cells, separated by SDS-PAGE, transferred to filters, and immunodecorated with either anti-HA or anti-Hsp60 serum as described in Materials and Methods. As a control (lane 3), proteins from nontransformed SCS1-null[a] cells grown at 30°C were also analyzed. Proteins were extracted from SCS1-null[a] cells expressing *SCS1*::*his*$_6$, and the Scs1 containing the C-terminal histidines was purified as described in Materials and Methods. This protein (lane 4) was separated by SDS-PAGE and stained with silver. As a control, proteins from SCS1-null[a] cells containing no plasmid were purified, subjected to SDS-PAGE, and analyzed in the same fashion (lane 5). (c) Localization of Scs1 by cellular fractionation. *SCS1*::*HA*$_3$*(2)*-expressing cells were grown overnight at 30°C. Spheroplasts were generated and cellular fractionation was carried out as described in Materials and Methods. Electrophoretically separated proteins were analyzed for the presence of Scs1 and Hsp60 as for panel b. Lane 1, total proteins extracted from cells before being converted to spheroplasts; lane 2, the initial homogenate of disrupted spheroplasts prior to any centrifugation; lane 3, purified mitochondria; lane 4, postmitochondrial supernatant. All four lanes contain identical cellular equivalents of protein.

the epitope-tagged Scs1 fully complemented the null allele. This finding suggests that the chimeric protein must be targeted to its normal cellular location and be fully functional.

Using a monoclonal antibody which recognizes the HA epitope, we immunodecorated Western blots of total proteins isolated from control cells and those expressing a single copy of *SCS1*::*HA*$_3$*(2)*. This analysis showed (Fig. 5b, lane 1) that a group of three or four immunologically reactive protein bands ranging in apparent mass from about 95 to 115 kDa was present in cells expressing *SCS1*::*HA*$_3$*(2)* but absent in control cells (Fig. 5b, lane 3). No forms of the immunoreactive proteins had the predicted size (ca. 89 kDa) of the fusion protein. This finding suggests that all Scs1 was modified in some manner and that differing degrees of modification occurred. Alternatively, the band with the lowest apparent mass could be an unmodified form of Scs1 with an anomalous electrophoretic mobility, with the other bands being the modified isoforms. The amounts and forms of these immunoreactive species were the same in cells growing at 30 and 37°C (Fig. 5b, lane 2), showing that Scs1 levels do not change in response to elevated temperature. The level of Hsp60 was increased about two- to threefold in the cells shifted to 37°C, indicating that a normal stress response occurred (22).

To determine whether the different electrophoretic forms were produced as a result of the presence of the C-terminal HA epitope, two other products of *SCS1* fusion genes were examined. The first was produced from a gene [*SCS1*::*HA*$_3$*(1)*] in which the HA_3 sequence was introduced at the internal *Bcl*I site (see Materials and Methods). The second was produced from a gene (*SCS1*::*his*$_6$), in which six histidine residues were added to the C terminus of Scs1. The first gene did not complement in single copy but did when expressed on a multicopy plasmid (data not shown). The latter complemented in single copy (Fig. 5a). The protein with the internal HA epitope was electrophoretically indistinguishable from that with the terminal HA epitope (data not shown), and the Scs1-His_6 protein purified by affinity chromatography had multiple forms of approximately the same sizes as the HA epitope fusion proteins (Fig. 5b). It is therefore likely that the multiplicity of forms is an inherent property of Scs1, but this cannot be confirmed until we have an antiserum to Scs1 itself.

The ability of *SCS1* to suppress a variety of *hsp60* mutant alleles indicates either a direct or an indirect role of Scs1 in the process of protein folding within mitochondria. If Scs1 is directly involved with Hsp60 function, one would expect it to be localized in the mitochondrial matrix or to the inner surface of the mitochondrial inner membrane. To test this, cells expressing a single copy of *SCS1*::*HA*$_3$*(2)* were subjected to subcellular fractionation. It was clear from this analysis (Fig. 5c) that Scs1 did not copurify with mitochondria but was recovered almost entirely in the postmitochondrial supernatant fraction. The small amount of Scs1 copurifying with mitochondria could indicate that a small fraction of Scs1 is normally targeted there. However, as it is the same size as the cytosolic protein, indicating no proteolytic processing typical of matrix-targeted proteins, we consider this alternative explanation unlikely but certainly not disproven. The fact that Scs1 did not copurify with Hsp60 makes it unlikely that it can be directly involved in processes within mitochondria.

While the multiple forms of Scs1 were evident when these fractionation experiments were carried out, reproducibly, we found that the relative amounts of the various forms differed from that seen when proteins were directly solubilized from unbroken cells. Until we know what posttranslational modifications are made to Scs1, we have no explanation for these findings.

The subcellular fractionation studies above were carried out on cells grown at 30°C. At it has been shown that some proteins can be redistributed within the cell at heat shock temperatures (35), we examined whether that might be the case for Scs1. Using indirect immunofluorescence, we determined the cellular location of epitope-tagged Scs1 in cells grown at 30 and

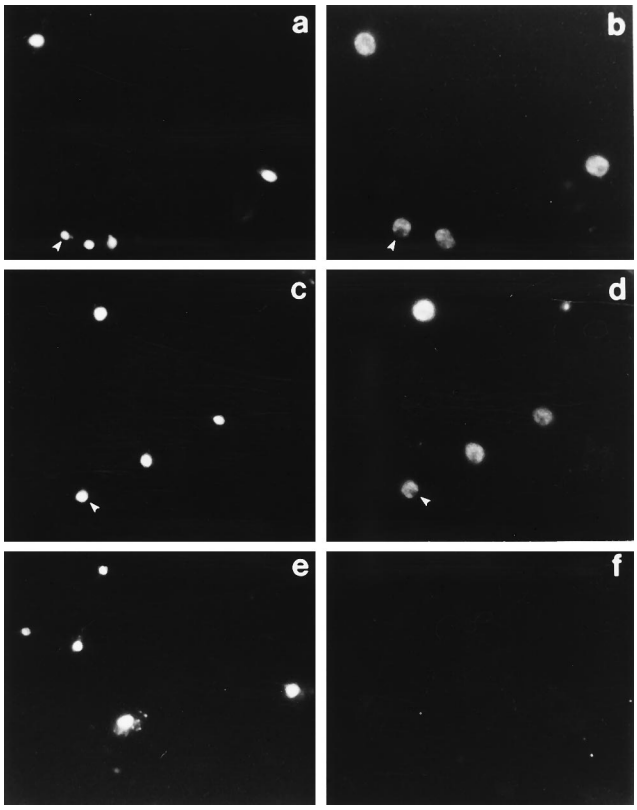

FIG. 6. Localization of Scs1 by immunocytochemistry. The same cells expressing *SCS1*::*HA*$_3$*(2)* as analyzed in Fig. 5 were grown at either 30°C (a and b) or 38°C (c and d) and prepared for immunocytochemical analysis as described in Materials and Methods. Fixed cells were treated with both 4′,6-diamidino-2-phenylindole (a, c, and e) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (b, d, and f). Control cells (e and f), the nontransformed SCS1-null[a] strain, were also grown at 30°C. The arrowheads indicate the positions of nuclei in comparable panels.

38°C (Fig. 6). At either temperature, Scs1 was located in the cytosol and excluded from the nucleus. If changes in Scs1 cellular location do occur as a result of increased temperatures, they were not detectable at this level of analysis.

Effects of deletion and overexpression of *SCS1* on the expression of other genes. Given that *SCS1*-null cells are *ts* for growth but Scs1 levels seem unaffected by temperature, and as Scs1 must indirectly affect Hsp60 function, we wondered whether Scs1 could be involved in regulating the levels of other mitochondrial chaperones. We first examined what effect the absence of Scs1 had on the levels of mRNAs for three mitochondrial chaperones, Hsp60, Cpn10 (28), and the mitochondrial GrpE homolog, Mge1 (2), when compared with mRNA levels in wild-type cells and in wild-type cells overexpressing *SCS1*. From the results shown in Fig. 7a, we concluded several things. First, in agreement with the Western analysis, the levels of expression of *SCS1* are not significantly different in wild-type cells at 30 and 38°C. Second, overexpression of *SCS1* in control cells (i.e., non-Hsp60 mutant strains) has no discernible effect on the levels of mRNAs produced from any of these genes at either temperature, with *HSP60* and *CPN10* showing their normal temperature inducibility (22, 28). However, the absence of Scs1 brought about a depression in the mRNA level encoding Mge1 at 30°C. More dramatically, for all three genes, little or no elevation in mRNA levels was found at 38°C in the SCS1-null[a] cells. For Hsp60 mRNA, there was actually a decline. Thus, the normal regulation of three mitochondrial chaperones apparently requires functional Scs1. By contrast, the synthesis of the major heat shock proteins in SCS1-null[a] cells at 37°C was qualitatively and quantitatively indistinguishable from that seen in wild-type cells (data not shown). Scs1 is clearly not required for the general heat shock response.

While no effect of overproducing Scs1 was seen for these three mRNAs in wild-type cells, it was conceivable that the effects of overexpression of *SCS1* might be different in *hsp60-ts* mutants. Examining the expression of the same three genes in ts3 cells containing one or multiple copies of *SCS1*, we found that to be the case (Fig. 7b). Overexpression of *SCS1* caused both Mge1 and Cpn10 mRNA levels to be higher than in wild-type cells at both 30 and 38°C, while there was no apparent differential effect relative to controls on Hsp60 mRNA levels at either temperature. The level of mRNA for mitochondrial Hsp70 (Scs1 [5]), another mitochondrial protein chaperone, appeared to be slightly elevated in cells overproducing Scs1 at 30°C, but no differences relative to controls were seen at 38°C. Overexpression of *SCS1* in ts3 cells had no discernible effects, compared with wild-type cells, on the normal levels of mRNAs for the large subunit of the coenzyme QH_2-cytochrome *c* reductase (Cor1 [34]), another mitochondrial protein, or on levels of the cytoplasmic protein actin or one of the large subunits of the vacuolar ATPase (VAT2 [36]). Although testing an admittedly limited array of genes, nonetheless, we found that only those encoding mitochondrial protein chaperones were affected by Scs1 overproduction.

DISCUSSION

We have identified an *S. cerevisiae* gene, *SCS1*, which, in high copy number, suppresses the lethal phenotype of a number of *hsp60-ts* alleles. As essentially all of our previous studies place Hsp60 solely within the mitochondrial matrix (11, 12, 22),

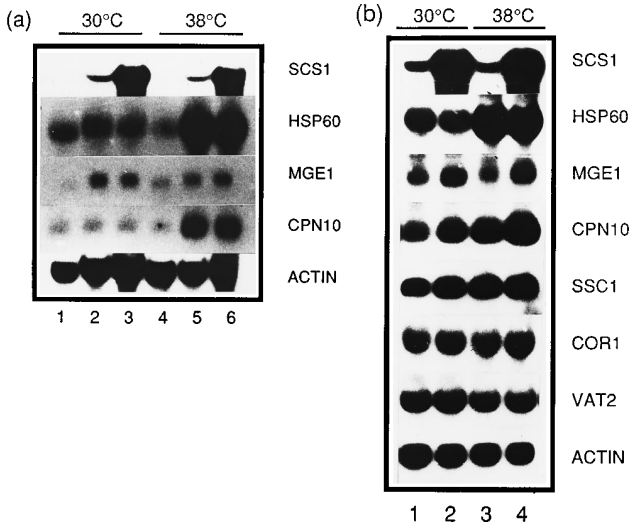

FIG. 7. Northern analyses of cells expressing different numbers of *SCS1* genes. RNA was isolated from cells grown at 30°C and from those grown at 30°C and then shifted to 38°C for 30 min. Northern analyses were carried out as described in Materials and Methods, using DNA probes encoding portions of the genes *SCS1*, *HSP60*, *CPN10*, *MGE1*, *SSC1*, *COR1*, and *VAT2*, and the actin gene. (a) Sources of RNA: SCS1-null[a] cells (lanes 1 and 4), W303 cells (lanes 2 and 5), and W303 cells transformed with YEpSCS1 (lane 3 and 6). Lanes 1 to 3, cells grown at 30°C; lanes 4 to 6, cells grown at 30°C and then shifted to 38°C. (b) Sources of RNA: ts3 cells (lanes 1 and 3) and ts3 cells transformed with the plasmid YEpSCS1. Lanes 1 and 2, cells grown at 30°C; lanes 3 and 4, cells grown at 30°C and then shifted to 38°C.

finding that Scs1 was not a mitochondrial protein was totally unexpected. However, this observation was consistent with the fact that overexpressed *SCS1* could not complement an *HSP60*-null allele and therefore did not suppress by replacing (i.e., bypassing) Hsp60 function. That is, overexpressed Scs1 must, in some manner, alleviate the misfunctioning of functionally abnormal Hsp60s. We considered that this might be achieved if high levels of Scs1 elevated Hsp60 levels, thereby increasing the overall activity of Hsp60s that are at least partially functional at nonpermissive temperatures. A direct test of that hypothesis proved it to be untenable. As there were no apparent quantitative or qualitative differences in the Hsp60s in the suppressed (or unsuppressed) mutant strains containing plasmid YEpSCS1, restoration of Hsp60 function would most likely have to occur through a functional or physical interaction with one or more other mitochondrial proteins whose levels and/or activities were affected by Scs1 overproduction.

The mutations in the alleles suppressed by Scs1 overproduction are scattered throughout the carboxyl-terminal one-half of Hsp60. It could be, however, that the domains containing the affected amino acids in the Hsp60 monomer share a common site within the normally folded native complex. As the altered amino acid residues in our mutant Hsp60s are conserved in the *E. coli* chaperonin GroEL (13, 25), we located the homologous sites in the recently published X-ray crystal structure of GroEL (3). The mutated residues were not localized at all, occurring in different regions of two well-separated domains of the GroEL monomer. These observations also suggest that in indirectly affecting Hsp60 function, Scs1 may act through a number of different intermediaries, not a single one.

The most likely known candidates for a direct or indirect functional interaction with Hsp60 are Cpn10 and Mge1 (10, 20). Cpn10 physically interacts with Hsp60 and functions as a cochaperone (10, 15). Similarly, Mge1 functions as a protein chaperone upstream of Hsp60 in the protein folding pathway of imported mitochondrial matrix proteins (2, 19, 21). Scs1-induced overexpression of one or the other, or both, of these genes could reasonably account for the suppressibility of a number of different mutant strains. One prediction is that if there are *hsp60-ts* mutants suppressible by overproduction of a single other molecular chaperone, they should be identifiable. We have begun such analyses by using the gene encoding the cochaperonin Cpn10. Thus far, in analyzing four mutant strains, we have not found one that is suppressible by Scs1 overproduction and also suppressible by Cpn10 overexpression. Another prediction is that we should see similar effects of overexpressing Scs1 on *MGE1* and *CPN10* expression in other *hsp60-ts* mutant strains. We have not yet examined any other strains to see whether that is true.

While evolutionary conservation of *SCS1* is reasonably high, no data regarding the functions of these homologs in higher plants or animals have been published. The only comparison available at present is that *SCS1* was previously identified as a multicopy suppressor of a mutation in the essential gene *ROX3* (27). *ROX3* encodes a nuclear regulatory factor that in some way controls, among other things, the production of the mitochondrial protein iso-2-cytochrome *c*. It is noteworthy that the gene encoding this protein, *CYC7*, is normally up-regulated at heat shock temperatures to a degree similar to that seen for other heat-inducible genes (24). Whether *ROX3* is involved in regulating other genes encoding mitochondrion-targeted proteins at either normal or heat shock temperatures has not been reported. We would predict that it might. How Scs1 overproduction suppresses the *ROX3* mutant and whether this is a direct or indirect effect is also not known. Preliminary data (37) indicate that the expression of *CYC7* is also altered in *SCS1*-null cells at both permissive and nonpermissive temperatures. Thus, another stress-inducible gene encoding a mitochondrion-targeted protein requires Scs1 for correct expression. From the data available thus far, we predict that Scs1 must be involved in some signaling pathway involving the expression of mitochondrion-targeted proteins during stress conditions.

While *SCS1* is not an essential gene and is not required for nonfermentative growth, it is absolutely required for growth and survival on all carbon sources at higher temperatures. It is also necessary for normal resistance to elevated levels of ethanol at 30°C. The levels of Scs1 are not normally elevated at higher temperatures, nor is there any indication that the transcription of *SCS1* is altered. As the *SCS1*-null strain grows more slowly in all media at 30°C, Scs1 must function at both normal and elevated temperatures. This conclusion is supported by the finding that the levels of Cpn10 and Mge1 mRNAs are considerably reduced in *SCS1*-null cells at 30°C. While it has not been proven that preventing the normal temperature-induced elevation of the molecular chaperones Hsp60, Cpn10, and Mge1 is necessarily lethal, it is certainly likely to be so. If this is the case, it could account for the *ts* lethality of *SCS1*-null cells.

We do not yet know what accounts for the anomalous electrophoretic patterns of Scs1 that we find when immunodecorating the proteins expressed from the *SCS1-HA$_3$* gene. While the three *SCS1* fusion genes expressed all produce heterogeneous arrays of proteins with similar sizes and patterns, we cannot conclude that this is the normal situation until we have produced an antibody against the normal Scs1. We did attempt to ascertain whether any of the various electrophoretic forms might be phosphorylated, glycosylated, or ubiquitinated. While the analyses were not exhaustive and were carried out on impure proteins, we obtained no evidence that any of these posttranslational modifications accounted in any way for the various electrophoretic isoforms. We also noted (data not shown) that the HA epitope-tagged Scs1 was highly unstable in vivo. Determination of whether this rapid turnover is intrinsic to Scs1 itself or is induced by the presence of the HA epitope will also require the availability of the proper antibodies.

ACKNOWLEDGMENTS

We especially thank Rich Zitomer for discussing his unpublished data regarding the *ROX3* suppressor. We also thank Liz Hallberg for helping to produce and characterize many of the strains used in this study. Haifeng Yang carried out some of the growth experiments with the *SCS1*-null cells. The individuals named in parentheses kindly supplied cloned copies of the following yeast genes: actin (Bob West), *COR1* (Mary Crivellone), *VAT2* (Patty Kane), *SSC1* (Betty Craig), and *MGE1* and *CPN10* (Sabine Rospert).

This work was supported by NIH grant GM-46302 to R.L.H.

ADDENDUM

Three different complete human cDNAs that are homologs of *SCS1* have now been cloned. They were shown to encode regulatory subunits (B) of protein phosphatase 2A (21a). While we have no direct evidence that Scs1 has the same function in *S. cerevisiae*, this observation is consistent with the fact that we find that overproduction of a regulatory subunit of the yeast Cdc28 kinase (Clb2) suppresses the *ts* phenotype of the *SCS1*-null strain.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, New York.
2. **Bolliger, L., O. Deloche, B. S. Glick, C. Georgopoulos, P. Jeno, N. Kronidou,**

M. Horst, N. Morishima, and G. Schatz. 1994. A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability. EMBO J. **13:**1998–2006.

3. **Braig, K., Z. Otwinowski, R. Hegde, D. C. Boisvert, A. Joachimiak, A. L. Horwich, and P. B. Sigler.** 1994. The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. Nature (London) **371:**578–586.
4. **Cheng, M. Y., F.-U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg, and A. L. Horwich.** 1989. Mitochondrial heat shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature (London) **337:**620–625.
5. **Craig, E. A., J. Kramern, J. Shilling, M. Werner-Washburne, S. Holmes, J. Kosic-Smithers, and C. M. Nicolet.** 1989. *SSC1*, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. Mol. Cell. Biol. **9:**3000–3008.
6. **Daum, G., P. C. Bohni, and G. Schatz.** 1982. Import of proteins into mitochondria: cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J. Biol. Chem. **257:**13028–13033.
7. **Davis, L. I., and G. R. Fink.** 1990. The *NUP1* gene encodes an essential component of the yeast nuclear pore complex. Cell **61:**965–978.
8. **Ellis, R. J., and S. M. van der Vies.** 1991. Molecular chaperones. Annu. Rev. Biochem. **60:**321–347.
9. **Engebrecht, J., J. Hirsch, and G. S. Roeder.** 1990. Meiotic gene conversion and crossing over: their relationship to each other and to chromosome synapsis and segregation. Cell **62:**927–937.
10. **Georgopoulos, C., K. Liberek, M. Zylicz, and D. Ang.** 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response, p. 209–249. *In* R. I. Morimoto, A. Tissieres, and C. Georgopolous (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. **Glick, B. S., A. Brandt, K. Cunningham, S. Muller, R. L. Hallberg, and G. Schatz.** 1992. Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast by a stop-transfer mechanism. Cell **69:**809–822.
12. **Hallberg, E. M., Y. Shu, and R. L. Hallberg.** 1993. Loss of mitochondrial hsp60 function: nonequivalent effects on matrix-targeted and intermembrane-targeted proteins. Mol. Cell. Biol. **13:**3050–3057.
13. **Hemmingson, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgeopoulos, R. W. Hendrix, and R. J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature (London) **333:**330–334.
14. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagaloff.** 1986. Yeast/E. coli shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
15. **Hohfield, J., and F.-U. Hartl.** 1994. Role of the chaperonin cofactor hsp10 in protein folding and sorting in yeast mitochondria. J. Cell Biol. **126:**305–315.
16. **Horwich, A. L., K. B. Low, W. A. Fenton, I. N. Hirshfield, and K. Furtak.** 1993. Folding in vivo of bacterial cytoplasmic proteins: role of groEL. Cell **74:**909–917.
17. **Kohrer, K., and H. Domdey.** 1991. Preparation of high molecular weight RNA. Methods Enzymol. **194:**398–405.
18. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
19. **Laloraya, S., B. D. Gambill, and E. A. Craig.** 1994. A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. Proc. Natl. Acad. Sci. USA **91:**6481–6485.
20. **Langer, T., C. Lu, E. Echols, J. Flanagan, M. K. Hayer, and F. U. Hartl.** 1992. Successive action of DnaK, DnaJ, and GroEL along the pathway of chaperone-mediated protein folding. Nature (London) **356:**683–689.
21. **Manning-Krieg, U., P. E. Scherer, and G. Schatz.** 1991. Sequential action of mitochondrial chaperonins in protein import into the matrix. EMBO J. **10:**3273–3280.

21a. **McCright, B., and D. M. Virshup.** Submitted for publication.

22. **McMullin, T. W., and R. L. Hallberg.** 1988. A highly evolutionarily conserved mitochondrial protein is structurally related to the protein encoded by the *Escherichia coli groEL* gene. Mol. Cell. Biol. **8:**371–380.
23. **Ostermann, J., A. L. Horwich, W. Neupert, and F.-U. Hartl.** 1989. Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. Nature (London) **341:**125–130.
24. **Pillar, T. M., and R. E. Bradshaw.** 1991. Heat shock and stationary phase induce transcription of the *Saccharomyces cerevisiae* iso-2 cytochrome *c* gene. Curr. Genet. **20:**185–188.
25. **Reading, D. S., R. L. Hallberg, and A. M. Myers.** 1989. Characterization of the yeast *HSP60* gene coding for a mitochondrial assembly factor. Nature (London) **337:**655–659.
26. **Rose, M. D., and G. R. Fink.** 1987. *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. Cell **48:**1047–1060.
27. **Rosenblum-Vos, L. S., L. Rhodes, C. C. Evangelista, K. A. Boayke, and R. S. Zitomer.** 1991. The *ROX3* gene encodes an essential nuclear protein involved in *CYC7* gene expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**5639–5647.
28. **Rospert, S., B. S. Glick, P. Jeno, G. Schatz, M. J. Todd, G. H. Lorimer, and P. Viitanen.** 1993. Identification and functional analysis of chaperonin 10, the gorES homolog from yeast mitochondria. Proc. Natl. Acad. Sci. USA **90:**10967–10971.
29. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–211.
30. **Rowley, N., C. Prip-Buus, B. Westerman, C. Brown, E. Schwarz, B. Barrell, and W. Neupert.** 1994. Mdj1p, a novel chaperone of the dnaJ family, is involved in mitochondrial biogenesis and protein folding. Cell **77:**249–259.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Schatz, P. J., F. Solomon, and D. Botstein.** 1988. Isolation and characterization of conditional-lethal mutations in the *TUB1* α-tubulin gene of the yeast *Saccharomyces cerevisiae*. Genetics **120:**681–695.

32a. **Shu, Y.** Unpublished data.

33. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
34. **Tzagaloff, A., M. Wu, and M. Crivellone.** 1986. Assembly of the mitochondrial membrane system. Characterization of COR1, the structural gene for the 44-kilodalton core protein of yeast coenzyme QH_2-cytochrome c reductase. J. Biol. Chem. **261:**17163–17169.
35. **Velazquez, J. M., and S. Lindquist.** 1984. Hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. Cell **36:**655–662.
36. **Yamashiro, C. T., P. M. Kane, D. F. Wolczyk, R. A. Preston, and T. H. Stevens.** 1990. Role of vacuolar acidification in protein sorting and zymogen activation: a genetic analysis of the yeast vacuolar proton-translocating ATPase. Mol. Cell. Biol. **10:**3737–3749.
37. **Zitomer, R.** Personal communication.